Abstract

Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione(CAS No. 8003-22-3), was tested for its ability to induce increase in the frequency of micronuclei in the bone marrow erythrocytes of mice. The test was dosed to mice orally at 2000, 666 and 222 mg/kg body weight in corn oil as a single dose(OECD 474 guidelines). Bone marrow smears were prepared from the test and negative control groups at 24 and 48 hours posttreatment. Bone marrow smears for the positive control group were prepared at 24 hours posttreatment. Polychromatic (PCE) and normochromatic (NCE) erythrocytes were scored from these bone marrow smears and were examined for the presence of micronuclei for evaluation of clastogenicity.There was no statistically significant increase in the frequency of micronucleated PCEs in the test substance dose groups as compared to the negative control group. The positive control group caused a statistically significant increase in micronucleated cells as compared to the negative control group. Based on the criteria of the study protocol, the test substance is considered nonclastogenic, under the experimental conditions

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14. ABSTRACT

Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione (CAS No. 8003-22-3), was tested for its ability to induce increase in the frequency of micronuclei in the bone marrow erythrocytes of mice. The test was dosed to mice orally at 2000, 666, and 222 mg/kg body weight in corn oil as a single dose (OECD 474 guidelines). Bone marrow smears were prepared from the test and negative control groups at 24 and 48 hours posttreatment. Bone marrow smears for the positive control group were prepared at 24 hours post treatment. Polychromatic (PCE) and normochromatic (NCE) erythrocytes were scored from these bone marrow smears and were examined for the presence of micronuclei for evaluation of clastogenicity. There was no statistically significant increase in the frequency of micronucleated PCEs in the test substance dose groups as compared to the negative control group. The positive control group caused a statistically signifiant increase in micronucleated cells as compared to the negative control group. Based on the criteria of the study protocol, the test substance is considered nonclastogenic, under the experimental conditions

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TOXIKON FINAL GLP REPORT: 10–3881–G1

RODENT BONE MARROW MICRONUCLEUS ASSAY

Test Substance
Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,

Author
Devaki Sadhu, Ph.D.

Final Report Date
January 18, 2011

COMPLIANCE
OECD Series on Principles of Good Laboratory Practice
And Compliance Monitoring
21 CFR, Part 58
Good Laboratory Practice for Non–Clinical Laboratory Studies

MANAGEMENT OF THE STUDY

Performing Laboratory
Toxikon Corporation
15 Wiggins Avenue
Bedford, MA 01730

Sponsor
U.S. Army Public Health Command
5158 Black Hawk Road
Aberdeen Proving Ground, MD 21010

Contract No.: W91ZLK-10-P-0886
Sponsor's Study Coordinator: Gunda Reddy, Ph.D., D.A.B.T.

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STUDY SUMMARY

The test substance, Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,, was tested for its ability to induce a statistically significant increase in the frequency of micronuclei in the bone marrow erythrocytes of mice. The test substance was dissolved in corn oil and administered to mice orally at 2000, 666 and 222 mg/kg body weight as a single treatment in accordance with the OECD 474 guidelines.

Bone marrow smears were prepared from the test and negative control groups at 24 and 48 hours post–treatment. Bone marrow smears for the positive control group were prepared at 24 hours post–treatment. Polychromatic (PCE) and normochromatic (NCE) erythrocytes were scored from these bone marrow smears and were examined for the presence of micronuclei for evaluation of clastogenicity.

There was no statistically significant increase in the frequency of micronucleated PCEs in the test substance dose groups as compared to the negative control group. The positive control group caused a statistically significant increase in micronucleated cells as compared to the negative control group. Based on the criteria of the study protocol, the test substance is considered non–clastogenic, under the experimental conditions.

QUALITY ASSURANCE STATEMENT

This study was conducted in compliance with U.S. Food and Drug Administration regulations set forth in 21 CFR, Part 58, and with the Organization for Economic Co–Operation and Development regulations set forth in OECD ENV/MC/CHEM(98)17, as revised in 1997.

The sections of the regulations not performed by or under the direction of Toxikon Corporation, exempt from this Good Laboratory Practice Statement, included characterization and stability of the test substance and its mixture with carriers, 21 CFR, Parts 58.105 and 58.113 and Part 6.2 of OECD ENV/MC/CHEM(98)17.

The Quality Assurance Unit conducted inspections on the following dates. The findings were reported to the Study Director and to Toxikon's Management.

INSPECTIONS	DATE OF INSPECTION	DATE REPORTED STUDY DIRECTOR	DATE REPORTED MANAGEMENT
SCORING	10/20/10	10/20/10	10/20/10
RAW DATA	01/18/11	01/18/11	01/18/11
DRAFT REPORT	01/18/11	01/18/11	01/18/11

Priti Patel, B.S.
Quality Assurance

1/18/11
Date

STUDY DIRECTOR SIGNATURE AND VERIFICATION DATES

This study meets the technical requirements of the protocol. The study also meets the requirements of the Good Laboratory Practice Regulations, 21 CFR, Part 58, and OECD GLPs, with the exemptions as stated in the Quality Assurance Statement.

Protocol Number:	P10–1759–00A
Study Director:	Devaki Sadhu, Ph.D.
Company:	Toxikon Corporation
Signature:	
Date:	01/18/11
Study Supervisor:	Devaki Sadhu, Ph.D.

VERIFICATION DATES:
The Study Initiation Date is the date the protocol is signed by the Study Director.

Test Substance Receipt:	08/18/10
Project Log Date:	08/30/10
Study Initiation Date:	09/07/10
Definitive Assay	
Technical Initiation	09/22/10
Technical Completion	11/04/10
Scoring:	09/22/10–11/04/10

1.0 PURPOSE

The purpose of this assay was to evaluate the potential of the test substance and/or its metabolites to induce micronuclei in maturing erythrocytes of mice. This procedure is designed to detect damage to the chromosomes or mitotic apparatus caused by the test substance.

2.0 REFERENCES

The study was based upon the following references:

2.1 OECD 474, Organization for Economic Co–Operation and Development (OECD), Guidelines for the Testing of Chemicals, "Mammalian Erythrocyte Micronucleus Test", adopted 21 July 1997.

2.2 ICH Harmonized Tripartite Guideline. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals, S2A, 1995, FDA: Published in the Federal Register, Vol. 61, April 24, 1996, page 18199.

2.3 ICH Harmonized Tripartite Guideline. Genotoxicity: A Standard Battery for Genotoxicity Testing for Pharmaceuticals, S2B, 1997, FDA: Published in the Federal Register, 21 November 1997.

2.4 Schmid, W. "The Micronucleus Test for Cytogenetic Analysis." Chemical Mutagens: Principles and Methods for their Detection. Vol 4. New York: Plenum Press, 1976. 31–53.

2.5 ISO/IEC 17025, 2005, General Requirements for the Competence of Testing and Calibration Laboratories.

3.0 COMPLIANCE

The study conformed to the current FDA 21 CFR, Part 58 – Good Laboratory Practice for Non–Clinical Laboratory Studies and OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring guidelines.

4.0 IDENTIFICATION OF TEST AND CONTROL SUBSTANCES

The following information was supplied by the Sponsor on a Test Requisition Form or other correspondence wherever applicable; it did not apply to confidential information. The Sponsor was responsible for all test substance characterization data and for providing a certificate of analysis.

4.1 Test Substance:

Test Substance Name: Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,
CAS/Code #: 8003-22-3
Lot/Batch #: 0704192/ DOD-D-51485A
Physical State: Solid
Color: Bright Lemon Yellow

Expiration Date: Not Supplied by Sponsor
Density: > 1
Stability: Stable at 4 °C
Solubility: Insoluble in water
pH: 7
Storage Conditions: Room Temperature
Safety Precautions: Standard Toxikon Laboratory Safety Precautions, Bovine source

4.2.1 Negative Control Substance: Corn Oil (CO)
Toxikon QC #: CSC–10–10–004–VV
Physical State: Liquid
Color: Colorless
Stability: Stable at Room Temperature
Storage Conditions: Room Temperature
Safety Precautions: Standard Laboratory Safety Precautions

4.2.2 Positive Control Substance Name: Cyclophosphamide (CP)
Toxikon QC #: LPR–10–09–013–GT
Physical State: Liquid
Color: Colorless
Stability: Stable
Storage Conditions: -20 ± 4 °C
Safety Precautions: Standard Laboratory Safety Precautions

5.0 IDENTIFICATION OF TEST SYSTEM

5.1 Animals Used in the Test:

Number and Species: 90 Swiss Albino mice (*Mus musculus*)

Sex: 45 males and 45 females (females were non–pregnant and nulliparous)

Weight/Age Range: 23.0–35.0 grams/at least 6 weeks old
weighed to the nearest 0.1 g

Health Status: healthy, not previously used in other experimental procedures

Animal Purchase: Hilltop Lab Animals, Scottdale, PA

Animal Identification: ear punch

Acclimation: minimum 5 days under the same conditions as for the actual test.

Animal Selection: selected from a larger pool of animals and examined to ensure lack of adverse clinical signs

5.2 Animal Care and Maintenance:

Animal Room Temperature: 68 ± 5 °F

Animal Room Relative Humidity: 30-70%

Air Exchanges per Hour: 10 to 15

Lights: 12-hour light/dark cycle, full spectrum fluorescent lights

Housing: group housing (5 per cage of same sex)

Cages: polycarbonate

Bedding: hardwood chips, P.W.I. Industries, St–Hyacinthe, Québec, Canada (contact)

Animal Rations: TEK 7012 Rodent Diet, Harlan Laboratories, Teklad, Madison, WI, *ad libitum*

Water: tap water, *ad libitum*

There were no known contaminants present in the feed, water, or bedding expected to interfere with the test data.

The laboratory and animal rooms were maintained as limited-access facilities

6.0 JUSTIFICATION OF TEST SYSTEM AND ROUTE OF ADMINISTRATION

Evaluation of induction of micronuclei in the bone marrow cells of mice has historically been used in detecting clastogenic activity of test materials and the guidelines have no alternative (non–animal) methods. The animal species, number, and routes of test substance administration are recommended in the references in Section 2.0.

7.0 EXPERIMENTAL DESIGN AND DOSAGE

7.1 Preparation of Test and Control Substances:

7.1.1 Test Substance:
The test substance was dissolved in corn oil and administered as specified by the Sponsor.

7.1.2 Positive Control Substance:
CP was employed as the positive control.

7.1.3 Negative Control Substance:
The negative control was Cottonseed Oil (CSO) for the range finding assay. Per Sponsor request, the main assay was conducted with Corn Oil as the negative control substance.

7.2 Pre–Dose Procedure:

7.2.1 Main Assay:
Animals were randomly assigned into treatment groups according to the following:

TABLE A
Definitive Assay Treatment Assignment

Group	Number of Animals	Timepoint
Test Substance (per dose)	10 (5 males and 5 females)	24 hours
	10 (5 males and 5 females)	48 hours
Negative Control	10 (5 males and 5 females)	24 hours
	10 (5 males and 5 females)	48 hours
Positive Control	10 (5 males and 5 females)	24 hours

7.2.2 Acclimated animals were weighed prior to dosing.

7.3 Dose Administration:

7.3.1 Frequency and Route of Test Substance Administration:
The test and control substances were administered *in vivo*, directly or through a solvent compatible with the test system by an appropriate route of administration.

The test and negative control substances were administered orally. The positive control substance was administered intraperitoneally. Test substances were administered as a single injection.

7.3.2 Dose Selection:
Per Sponsor request and the OECD guidelines, the definitive assay was conducted using 2000, 666, and 222 mg/kg concentrations of the test substance in corn oil. The dosing volume of the test substance solution was 20 mL per kg body weight.

7.3.3 Control Substance:
Per Sponsor request, corn oil which was used to dissolve the test substance served as the negative control substance. The control substance was administered by the same route at a volume of 20 mL/kg body weight. The positive control was administered intraperitoneally at a dose of 25 µg/g body weight.

7.4 Post–Dose Procedure:

7.4.1 Clinical observations were conducted daily.

7.4.2 Animals from the test, negative, and positive control groups were sacrificed 24 hours after the treatment. At 48 hours after the treatment, animals from the test substance concentration and negative control group were sacrificed. The animals were euthanized by CO_2 inhalation.

7.4.3 At each sacrifice interval, bone marrow slides were prepared. Immediately after sacrifice, one or both femurs were exposed by appropriate surgical techniques. The bone marrow was collected and placed on a clean, pre–labeled microscope slide. Using a thick plastic cover glass, a fine feather–edge smear was prepared. The slides were then dried at room temperature, fixed in methanol, and stained with Giemsa.

7.4.4 The animals were weighed prior to terminal sacrifice.

7.4.5 Scoring:
The treatment and negative control slides were coded prior to scoring to reduce any possible bias. A total of at least 2000 polychromatic erythrocytes (PCEs) per animal were scored for the presence of micronuclei. The scored elements are the number of micronucleated cells, and not the number of micronuclei. The ratio of polychromatic to normochromatic erythrocytes were also determined by counting the appropriate number of normochromatic erythrocytes per 2000 polychromatic cells.

8.0 EVALUATION CRITERIA

8.1 Evaluation:
The number of micronucleated polychromatic erythrocytes for the positive and negative controls should be in the range of expected historical control values. The frequency of micronucleated PCEs in the positive control group should be statistically significantly greater than the negative control group. If these conditions are not met the test should be repeated.

8.2 Statistical Analysis:
Data are analyzed separately for male and female animals. The frequency of micronucleated PCEs in each dose group is compared to that in the respective negative control and to each other using a program such as the "t–test" using Graph Pad Prism Software by Analytical Software, Inc. (Analyzing Data with Graph Pad Prism®, Harvey Motulsky). This statistical method determines if there is a significant ($p \leq 0.05$) increase in the incidence of micronucleated cells in the test substance group as compared to the negative control group. A dose related response is determined, if appropriate, by a linear regression analysis. Biological and statistical significance is considered in the evaluation. Differences are considered not significant with $p > 0.05$.

8.3 Positive Dose Response:

8.3.1 Single Test Dose:
The test substance is considered to have caused a positive response in the assay if a reproducible and statistically significant increase in micronucleated polychromatic erythrocytes is observed compared to concurrent negative control.

8.3.2 Multiple Test Doses:
For a study involving multiple dose levels, the test substance is considered to have caused a positive response in the assay if at least one test substance dose exhibits a reproducible statistically significant increase ($p \leq 0.05$) in micronucleated polychromatic erythrocytes over its concurrent negative control.

Alternately, the test substance is considered to have caused a positive dose response in the assay if a dose–related increase in the number of micronucleated polychromatic erythrocytes with $r \geq 0.95$ (obtained from the Linear Regression data analysis) is observed. Statistical and biological significance will be taken into consideration in the evaluation of results.

8.4 Confirmatory Assay (Optional):
A confirmatory assay is performed when results of the assay are equivocal. The confirmatory assay is performed under conditions as specified by the Sponsor. Conditions of the confirmatory assay, if requested, are added as an amendment to the protocol.

A confirmatory assay is performed when requested by the Sponsor.

8.5 The study and its design employ methodology to minimize uncertainty of measurement and control of bias for data collection and analysis.

9.0 RESULTS

9.1 Micronucleus Assay:

9.1.1 Animal Weights and Clinical Observations

9.1.1.1 Test Substance Dose Groups (Tables 3 - 8):
None of the animals showed any clinical signs of toxicity. All animals gained weight at the time of sacrifice.

9.1.1.2 Control Dose Groups (Tables 1, 2, and 9):
All animals gained weight at the time of sacrifice.

9.2 Micronucleus Scoring (Tables 1–9):

9.2.1 There was a statistically significant increase in the number of micronucleated cells in the positive control group, as compared to the negative control group, thus validating the conduct of the assay.

9.2.2 There was no statistically significant increase in the number of micronucleated cells in the test substance groups at either time points, as compared to the concurrent negative control groups.

10.0 CONCLUSION

The test substance, Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,, was tested for its ability to induce a statistically significant increase in the frequency of micronuclei in the bone marrow erythrocytes of mice. The test substance was dissolved in corn oil and administered to mice orally at 2000, 666 and 222 mg/kg body weight as a single treatment in accordance with the OECD 474 guidelines.

Bone marrow smears were prepared from the test and negative control groups at 24 and 48 hours post–treatment. Bone marrow smears for the positive control group were prepared at 24 hours

post–treatment. Polychromatic (PCE) and normochromatic (NCE) erythrocytes were scored from these bone marrow smears and were examined for the presence of micronuclei for evaluation of clastogenicity.

There was no statistically significant increase in the frequency of micronucleated PCEs in the test substance dose groups as compared to the negative control group. The positive control group caused a statistically significant increase in micronucleated cells as compared to the negative control group. Based on the criteria of the study protocol, the test substance is considered non–clastogenic, under the experimental conditions.

11.0 RECORDS

11.1 Original raw data is archived at Toxikon Corporation.

11.2 A copy of the final report and any report amendments is archived at Toxikon Corporation.

11.3 The original final report and a copy of any protocol amendments or deviations is forwarded to the Sponsor.

11.4 All used and unused test substance shall be disposed of by Toxikon per Sponsor's request.

12.0 CONFIDENTIALITY AGREEMENT

Per corporate policy, confidentiality shall be maintained in general, and in specific accordance with any relevant agreement specifically executed between Toxikon and the Sponsor.

13.0 ANIMAL WELFARE STATEMENT

The Sponsor assured that, to the best of their knowledge, this study did not unnecessarily duplicate previous testing and that there were no non–animal alternatives acceptable for the evaluation of this test substance as defined by the protocol.

No evidence of pain and suffering was reported to the Veterinarian and/or Study Director.

Toxikon strictly adhered to the following standards in maintaining the animal care and use program:

United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service, 9 CFR Ch. 1 (1/1/95 edition), Subchapter A–Animal Welfare.

"Guide for the Care and Use of Laboratory Animals," National Research Council, 1996. (NIH).

Office for Laboratory Animal Welfare (OLAW), "Public Health Service Policy on Humane Care and Use of Laboratory Animals," Health Research Extension Act of 1985 (Public Law 99–158 November 20, 1985), Reprinted 1996.

ISO 10993–2, 2006, Biological Evaluation of Medical Devices – Part 2: Animal Welfare Requirements.

Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

14.0 PROTOCOL AMENDMENT

The protocol describes range finding assay in Sections 5.1 and 7.2. Per Sponsor specification, the main assay was conducted with corn oil as the solvent using the highest recommended dose of 2000 mg/kg body weight followed by two lower doses of 666 and 222 mg/kg body weight per OECD guidelines without a range finding study.

This change did not have any impact on the outcome of the study as the necessary doses required by the guidelines have been tested and satisfactory data have been obtained.

TABLE 1
Negative Control (24 hours)
Body Weights, Dosing Data, Clinical Observations, and Micronucleus Scoring Data

Test Substance: Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,

Lot/Batch #: 0704192/ DOD-D-51485A

Animal #/Sex	# PCE	Negative Control (24 hours)			MNC / PCE
		# NCE	PCE / NCE	# MNC	
DA61/Male	2000	1964	1.0183	3	0.0015
DA62/Male	2000	1978	1.0111	3	0.0015
DA63/Male	2000	1883	1.0621	2	0.0010
DA64/Male	2000	2006	0.9970	2	0.0010
DA65/Male	2000	1943	1.0293	3	0.0015
Average	**2000**	**1955**	**1.0236**	**3**	**0.0013**
SD	**0**	**46**	**0.0245**	**1**	**0.0003**
DA66/Male	2000	1928	1.0373	2	0.0010
DA67/Male	2000	2010	0.9950	2	0.0010
DA68/Male	2000	2137	0.9359	2	0.0010
DA69/Male	2000	1922	1.0406	3	0.0015
DA70/Male	2000	1893	1.0565	3	0.0015
Average	**2000**	**1978**	**1.0131**	**2**	**0.0012**
SD	**0**	**99**	**0.0488**	**1**	**0.0003**

Animal #/Sex	Body Weight (g)			Dose (mL)*	Clinical Observations**
	Day 0 (10/14/410)	Day 1 (10/15/10)	Weight Change		
DA61/Male	27.1	27.9	0.8	0.54	Normal
DA62/Male	24.1	25.2	1.1	0.48	Normal
DA63/Male	25.1	26.2	1.1	0.50	Normal
DA64/Male	26.9	27.5	0.6	0.54	Normal
DA65/Male	24.1	24.7	0.6	0.48	Normal
Average	**25.5**	**26.3**	**0.8**	**0.5**	
SD	**1.5**	**1.4**	**0.3**	**0.0**	
DA66/Male	28.1	28.9	0.8	0.56	Normal
DA67/Male	28.7	29.6	0.9	0.57	Normal
DA68/Male	28.9	29.9	1.0	0.58	Normal
DA69/Male	25.2	27.0	1.8	0.50	Normal
DA70/Male	25.4	26.1	0.7	0.51	Normal
Average	**27.3**	**28.3**	**1.0**	**0.5**	
SD	**1.8**	**1.7**	**0.4**	**0.0**	

PCE = Polychromatic Erythrocytes
NCE = Normochromatic Erythrocytes
DA = Definitive Assay Animal
MNC = Micronucleated Cells
SD = Standard Deviation
* = Negative Control Substance dosed at 20 mL/kg body weight
** = Summary of Clinical Observations, Day 0 through Day 1

TABLE 2
Negative Control (48 hours)
Body Weights, Dosing Data, Clinical Observations, and Micronucleus Scoring Data

Test Substance: Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,

Lot/Batch #: 0704192/ DOD-D-51485A

Animal #/Sex	# PCE	Negative Control (48 hours)			MNC / PCE
		# NCE	PCE / NCE	# MNC	
DA71/Female	2000	2027	0.9867	2	0.0010
DA72/Female	2000	1951	1.0251	4	0.0020
DA73/Female	2000	1905	1.0499	3	0.0015
DA74/Female	2000	1966	1.0173	3	0.0015
DA75/Female	2000	2122	0.9425	2	0.0010
Average	**2000**	**1796**	**1.0043**	**3**	**0.0014**
SD	**0**	**84**	**0.0413**	**1**	**0.0004**
DA76/Male	2000	1796	1.1136	2	0.0010
DA77/Male	2000	1934	1.0341	4	0.0020
DA78/Male	2000	1876	1.0661	3	0.0015
DA79/Male	2000	2001	0.9995	3	0.0015
DA80/Male	2000	1988	1.0060	2	0.0010
Average	**2000**	**1919**	**1.0439**	**3**	**0.0014**
SD	**0**	**85**	**0.0470**	**1**	**0.0004**

Animal #/Sex	Body Weight (g)			Dose (mL)*	Clinical Observations**
	Day 0 (10/14/140)	Day 2 (10/15/10)	Weight Change		
DA71/Female	31.2	32.4	1.2	0.62	Normal
DA72/Female	29.2	30.5	1.3	0.58	Normal
DA73/Female	28.4	29.6	1.2	0.57	Normal
DA74/Female	29.1	30.4	1.3	0.58	Normal
DA75/Female	29.1	30.1	1.0	0.58	Normal
Average	**29.4**	**30.6**	**1.2**	**0.6**	
SD	**1.1**	**1.1**	**0.1**	**0.0**	
DA76/Male	27.6	28.2	0.6	0.55	Normal
DA77/Male	27.8	28.6	0.8	0.56	Normal
DA78/Male	27.3	28.1	0.8	0.55	Normal
DA79/Male	28.6	29.2	0.6	0.57	Normal
DA80/Male	23.1	24.2	1.1	0.46	Normal
Average	**26.9**	**27.7**	**0.8**	**0.5**	
SD	**2.2**	**2.0**	**0.2**	**0.0**	

PCE = Polychromatic Erythrocytes
NCE = Normochromatic Erythrocytes
DA = Definitive Assay Animal
MNC = Micronucleated Cells
SD = Standard Deviation
* = Negative Control Substance dosed at 20 mL/kg body weight
** = Summary of Clinical Observations, Day 0 through Day 2

TABLE 3
Test Substance: 2000 mg/kg Body Weight (24 hours)
Body Weights, Dosing Data, Clinical Observations, and Micronucleus Scoring Data

Test Substance: Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,

Lot/Batch #: 0704192/ DOD-D-51485A

Animal #/Sex	# PCE	Test Substance (24 hours)			MNC / PCE
		# NCE	PCE / NCE	# MNC	
DA1/Male	2000	1959	1.0209	2	0.0010
DA2/Male	2000	2003	0.9985	3	0.0015
DA3/Male	2000	1905	1.0499	4	0.0020
DA4/Male	2000	1976	1.0121	3	0.0015
DA5/Male	2000	1892	1.0571	3	0.0015
Average	**2000**	**1947**	**1.0277**	**3**	**0.0015**
SD	**0**	**47**	**0.0250**	**1**	**0.0004**
DA6/Female	2000	1897	1.0543	4	0.0020
DA7/Female	2000	2016	0.9921	3	0.0000
DA8/Female	2000	2165	0.9238	3	0.0000
DA9/Female	2000	2047	0.9770	3	0.0015
DA10/Female	2000	1986	1.0070	2	0.0010
Average	**2000**	**2022**	**0.9908**	**3**	**0.0009**
SD	**0**	**98**	**0.0474**	**1**	**0.0009**

Animal #/Sex	Body Weight (g)			Dose (mL)*	Clinical Observations**
	Day 0 (10/14/10)	Day 1 (10/15/10)	Weight Change		
DA1/Male	28.2	28.9	0.7	0.56	Normal
DA2/Male	24.6	25.8	1.2	0.49	Normal
DA3/Male	23.7	24.9	1.2	0.47	Normal
DA4/Male	29.2	30.5	1.3	0.58	Normal
DA5/Male	24.7	25.3	0.6	0.49	Normal
Average	**26.1**	**27.1**	**1.0**	**0.5**	
SD	**2.4**	**2.5**	**0.3**	**0.0**	
DA6/Female	26.7	27.0	0.3	0.53	Normal
DA7/Female	28.1	28.7	0.6	0.56	Normal
DA8/Female	24.2	25.5	1.3	0.48	Normal
DA9/Female	28.2	29.9	1.7	0.56	Normal
DA10/Female	28.9	30.7	1.8	0.58	Normal
Average	**27.2**	**28.4**	**1.1**	**0.5**	
SD	**1.9**	**2.1**	**0.7**	**0.0**	

PCE = Polychromatic Erythrocytes
NCE = Normochromatic Erythrocytes
MNC = Micronucleated Cells
SD = Standard Deviation
DA = Definitive Assay Animal
* = Test Substance administered at 20 mL/kg body weight
** = Summary of Clinical Observations, Day 0 through Day 1

TABLE 4
Test Substance: 2000 mg/kg Body Weight (48 hours)
Body Weights, Dosing Data, Clinical Observations, and Micronucleus Scoring Data

Test Substance: Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,

Lot/Batch #: 0704192/ DOD-D-51485A

Animal #/Sex	# PCE	Test Substance (48 hours)			MNC / PCE
		# NCE	PCE / NCE	# MNC	
DA11/Male	2000	1950	1.0256	3	0.0015
DA12/Male	2000	2067	0.9676	2	0.0010
DA13/Male	2000	2101	0.9519	3	0.0015
DA14/Male	2000	1949	1.0262	4	0.0020
DA15/Male	2000	2006	0.9970	3	0.0015
Average	**2000**	**2015**	**0.9937**	**3**	**0.0015**
SD	**0**	**68**	**0.0336**	**1**	**0.0004**
DA16/Female	2000	1955	1.0230	3	0.0015
DA17/Female	2000	2136	0.9363	3	0.0000
DA18/Female	2000	2082	0.9606	4	0.0000
DA19/Female	2000	1979	1.0106	2	0.0010
DA20/Female	2000	1968	1.0163	3	0.0015
Average	**2000**	**2024**	**0.9894**	**3**	**0.0008**
SD	**0**	**80**	**0.0386**	**1**	**0.0008**

Animal #/Sex	Body Weight (g)			Dose (mL)*	Clinical Observations**
	Day 0 (05/19/10)	Day 2 (05/21/10)	Weight Change		
DA11/Male	28.3	29.2	0.9	0.57	Normal
DA12/Male	28.3	29.6	1.3	0.57	Normal
DA13/Male	27.2	28.4	1.2	0.54	Normal
DA14/Male	25.6	26.1	0.5	0.51	Normal
DA15/Male	27.1	27.9	0.8	0.54	Normal
Average	**27.3**	**28.2**	**0.9**	**0.5**	
SD	**1.1**	**1.4**	**0.3**	**0.0**	
DA16/Female	29.0	30.4	1.4	0.58	Normal
DA17/Female	24.2	25.8	1.6	0.48	Normal
DA18/Female	29.6	29.7	0.1	0.59	Normal
DA19/Female	24.3	25.6	1.3	0.49	Normal
DA20/Female	29.2	30.2	1.0	0.58	Normal
Average	**27.3**	**28.3**	**1.1**	**0.5**	
SD	**2.8**	**2.4**	**0.6**	**0.1**	

PCE = Polychromatic Erythrocytes
NCE = Normochromatic Erythrocytes
MNC = Micronucleated Cells
SD = Standard Deviation
DA = Definitive Assay Animal
* = Test Substance administered at 20 mL/kg body weight
** = Summary of Clinical Observations, Day 0 through Day 2

TABLE 5
Test Substance: 666 mg/kg Body Weight (24 hours)
Body Weights, Dosing Data, Clinical Observations, and Micronucleus Scoring Data

Test Substance: Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,

Lot/Batch #: 0704192/ DOD-D-51485A

Animal #/Sex	# PCE	Test Substance (48 hours)			MNC / PCE
		# NCE	PCE / NCE	# MNC	
DA21/Male	2000	1937	1.0325	3	0.0015
DA22/Male	2000	1896	1.0549	3	0.0015
DA23/Male	2000	2015	0.9926	4	0.0020
DA24/Male	2000	2146	0.9320	2	0.0010
DA25/Male	2000	2005	0.9975	2	0.0010
Average	**2000**	**2000**	**1.0019**	**3**	**0.0014**
SD	**0**	**95**	**0.0467**	**1**	**0.0004**
DA26/Female	2000	1996	1.0020	3	0.0015
DA27/Female	2000	1953	1.0241	2	0.0000
DA28/Female	2000	1978	1.0111	3	0.0000
DA29/Female	2000	2115	0.9456	4	0.0020
DA30/Female	2000	2076	0.9634	2	0.0010
Average	**2000**	**2024**	**0.9892**	**3**	**0.0009**
SD	**0**	**69**	**0.0333**	**1**	**0.0009**

Animal #/Sex	Body Weight (g)			Dose (mL)*	Clinical Observations**
	Day 0 (10/14/10)	Day 2 (10/15/10)	Weight Change		
DA21/Male	30.2	31.3	1.1	0.60	Normal
DA22/Male	24.6	24.9	0.3	0.49	Normal
DA23/Male	23.9	24.7	0.8	0.48	Normal
DA24/Male	24.6	25.2	0.6	0.49	Normal
DA25/Male	29.2	30.6	1.4	0.58	Normal
Average	**26.5**	**27.3**	**0.8**	**0.5**	
SD	**3.0**	**3.3**	**0.4**	**0.1**	
DA26/Female	29.2	30.4	1.2	0.58	Normal
DA27/Female	27.4	28.1	0.7	0.55	Normal
DA28/Female	25.9	26.9	1.0	0.52	Normal
DA29/Female	28.4	30.1	1.7	0.57	Normal
DA30/Female	26.2	26.9	0.7	0.52	Normal
Average	**27.4**	**28.5**	**1.1**	**0.5**	
SD	**1.4**	**1.7**	**0.4**	**0.0**	

PCE = Polychromatic Erythrocytes
NCE = Normochromatic Erythrocytes
MNC = Micronucleated Cells
SD = Standard Deviation
DA = Definitive Assay Animal
* = Test Substance administered at 20 mL/kg body weight
** = Summary of Clinical Observations, Day 0 through Day 2

TABLE 6
Test Substance: 666 mg/kg Body Weight (48 hours)
Body Weights, Dosing Data, Clinical Observations, and Micronucleus Scoring Data

Test Substance: Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,

Lot/Batch #: 0704192/ DOD-D-51485A

Animal #/Sex	# PCE	Test Substance (48 hours)			MNC / PCE
		# NCE	PCE / NCE	# MNC	
DA31/Male	2000	1956	1.0225	3	0.0015
DA32/Male	2000	2035	0.9828	3	0.0015
DA33/Male	2000	1905	1.0499	2	0.0010
DA34/Male	2000	2147	0.9315	4	0.0020
DA35/Male	2000	2022	0.9891	3	0.0015
Average	**2000**	**2013**	**0.9952**	**3**	**0.0015**
SD	**0**	**91**	**0.0447**	**1**	**0.0004**
DA36/Female	2000	1978	1.0111	3	0.0015
DA37/Female	2000	1965	1.0178	2	0.0000
DA38/Female	2000	1896	1.0549	3	0.0000
DA39/Female	2000	2103	0.9510	4	0.0020
DA40/Female	2000	2037	0.9818	3	0.0015
Average	**2000**	**1996**	**1.0033**	**3**	**0.0010**
SD	**0**	**78**	**0.0391**	**1**	**0.0009**

Animal #/Sex	Body Weight (g)			Dose (mL)*	Clinical Observations**
	Day 0 (10/14/10)	Day 2 (10/15/10)	Weight Change		
DA31/Male	25.0	26.3	1.3	0.50	Normal
DA32/Male	26.4	27.2	0.8	0.53	Normal
DA33/Male	25.8	26.4	0.6	0.52	Normal
DA34/Male	29.8	30.5	0.7	0.60	Normal
DA35/Male	28.2	29.5	1.3	0.56	Normal
Average	**27.0**	**28.0**	**0.9**	**0.5**	
SD	**1.9**	**1.9**	**0.3**	**0.0**	
DA36/Female	31.1	32.7	1.6	0.62	Normal
DA37/Female	27.2	28.4	1.2	0.54	Normal
DA38/Female	24.5	25.3	0.8	0.49	Normal
DA39/Female	25.6	26.7	1.1	0.51	Normal
DA40/Female	26.8	27.2	0.4	0.54	Normal
Average	**27.0**	**28.1**	**1.0**	**0.5**	
SD	**2.5**	**2.8**	**0.4**	**0.0**	

PCE = Polychromatic Erythrocytes MNC = Micronucleated Cells
NCE = Normochromatic Erythrocytes SD = Standard Deviation
DA = Definitive Assay Animal
* = Test Substance administered at 20 mL/kg body weight
** = Summary of Clinical Observations, Day 0 through Day 2

TABLE 7
Test Substance: 222 mg/kg Body Weight (24 hours)
Body Weights, Dosing Data, Clinical Observations, and Micronucleus Scoring Data

Test Substance: Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,

Lot/Batch #: 0704192/ DOD-D-51485A

Animal #/Sex	# PCE	Test Substance (48 hours)			MNC / PCE
		# NCE	PCE / NCE	# MNC	
DA41/Male	2000	1998	1.0010	3	0.0015
DA42/Male	2000	2145	0.9324	4	0.0020
DA43/Male	2000	1967	1.0168	3	0.0015
DA44/Male	2000	1895	1.0554	2	0.0010
DA45/Male	2000	1907	1.0488	2	0.0010
Average	**2000**	**1982**	**1.0109**	**3**	**0.0014**
SD	**0**	**100**	**0.0493**	**1**	**0.0004**
DA46/Female	2000	1968	1.0163	3	0.0015
DA47/Female	2000	2011	0.9945	2	0.0000
DA48/Female	2000	1842	1.0858	4	0.0000
DA49/Female	2000	1960	1.0204	3	0.0015
DA50/Female	2000	1899	1.0532	4	0.0020
Average	**2000**	**1936**	**1.0340**	**3**	**0.0010**
SD	**0**	**66**	**0.0357**	**1**	**0.0009**

Animal #/Sex	Body Weight (g)			Dose (mL)*	Clinical Observations**
	Day 0 (10/14/10)	Day 2 (10/15/10)	Weight Change		
DA41/Male	24.9	25.5	0.6	0.50	Normal
DA42/Male	25.4	26.1	0.7	0.51	Normal
DA43/Male	25.2	25.9	0.7	0.50	Normal
DA44/Male	28.7	29.7	1.0	0.57	Normal
DA45/Male	26.1	26.9	0.8	0.52	Normal
Average	**26.1**	**26.8**	**0.8**	**0.5**	
SD	**1.5**	**1.7**	**0.2**	**0.0**	
DA46/Female	30.1	31.0	0.9	0.60	Normal
DA47/Female	30.3	30.9	0.6	0.61	Normal
DA48/Female	26.4	27.2	0.8	0.53	Normal
DA49/Female	26.2	27.4	1.2	0.52	Normal
DA50/Female	27.1	27.8	0.7	0.54	Normal
Average	**28.0**	**28.9**	**0.8**	**0.6**	
SD	**2.0**	**1.9**	**0.2**	**0.0**	

PCE = Polychromatic Erythrocytes
NCE = Normochromatic Erythrocytes
MNC = Micronucleated Cells
SD = Standard Deviation
DA = Definitive Assay Animal
* = Test Substance administered at 20 mL/kg body weight
** = Summary of Clinical Observations, Day 0 through Day 2

TABLE 8
Test Substance: 222 mg/kg Body Weight (48 hours)
Body Weights, Dosing Data, Clinical Observations, and Micronucleus Scoring Data

Test Substance: Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,

Lot/Batch #: 0704192/ DOD-D-51485A

Animal #/Sex	# PCE	Test Substance (48 hours)			MNC / PCE
		# NCE	PCE / NCE	# MNC	
DA51/Male	2000	2044	0.9785	3	0.0015
DA52/Male	2000	2133	0.9376	4	0.0020
DA53/Male	2000	1958	1.0215	3	0.0015
DA54/Male	2000	2007	0.9965	3	0.0015
DA55/Male	2000	1933	1.0347	2	0.0010
Average	**2000**	**2015**	**0.9937**	**3**	**0.0015**
SD	**0**	**79**	**0.0382**	**1**	**0.0004**
DA56/Female	2000	1803	1.1093	2	0.0010
DA57/Female	2000	1947	1.0272	2	0.0000
DA58/Female	2000	1961	1.0199	3	0.0000
DA59/Female	2000	2103	0.9510	2	0.0010
DA60/Female	2000	2033	0.9838	3	0.0015
Average	**2000**	**1969**	**1.0182**	**2**	**0.0007**
SD	**0**	**112**	**0.0593**	**1**	**0.0007**

Animal #/Sex	Body Weight (g)			Dose (mL)*	Clinical Observations**
	Day 0 (10/14/10)	Day 2 (10/15/10)	Weight Change		
DA51/Male	26.6	27.3	0.7	0.53	Normal
DA52/Male	30.1	31.4	1.3	0.60	Normal
DA53/Male	28.7	29.7	1.0	0.57	Normal
DA54/Male	27.1	28.6	1.5	0.54	Normal
DA55/Male	24.6	25.2	0.6	0.49	Normal
Average	**27.4**	**28.4**	**1.0**	**0.5**	
SD	**2.1**	**2.4**	**0.4**	**0.0**	
DA56/Female	27.2	28.3	1.1	0.54	Normal
DA57/Female	27.0	27.9	0.9	0.54	Normal
DA58/Female	28.9	29.6	0.7	0.58	Normal
DA59/Female	28.0	29.3	1.3	0.56	Normal
DA60/Female	26.5	27.8	1.3	0.53	Normal
Average	**27.5**	**28.6**	**1.1**	**0.6**	
SD	**0.9**	**0.8**	**0.3**	**0.0**	

PCE = Polychromatic Erythrocytes
NCE = Normochromatic Erythrocytes
MNC = Micronucleated Cells
SD = Standard Deviation
DA = Definitive Assay Animal
* = Test Substance administered at 20 mL/kg body weight
** = Summary of Clinical Observations, Day 0 through Day 2

TABLE 9
Positive Control: Cyclophosphamide 25 µg/g (24 hours)
Body Weights, Dosing Data, Clinical Observations, and Micronucleus Scoring Data

Test Substance: Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,

Lot/Batch #: 0704192/ DOD-D-51485A

Animal #/Sex	# PCE	Positive Control (24 hours)			MNC / PCE	
		# NCE	PCE / NCE	# MNC		
DA81/Male	2000	1976	1.0121	30	0.0150	
DA82/Male	2000	2102	0.9515	27	0.0135	
DA83/Male	2000	2036	0.9823	22	0.0110	
DA84/Male	2000	2107	0.9492	28	0.0140	
DA85/Male	2000	1933	1.0347	25	0.0125	
Average	**2000**	**2031**	**0.9860**	**26**	**0.0132**	@
SD	**0**	**77**	**0.0375**	**3**	**0.0015**	
DA86/Female	2000	1980	1.0101	21	0.0105	
DA87/Female	2000	1960	1.0204	23	0.0115	
DA88/Female	2000	2079	0.9620	27	0.0135	
DA89/Female	2000	1897	1.0543	22	0.0110	
DA90/Female	2000	1942	1.0299	31	0.0155	
Average	**2000**	**1972**	**1.0153**	**25**	**0.0124**	@
SD	**0**	**67**	**0.0340**	**4**	**0.0021**	

Animal #/Sex	Body Weight (g)			Dose (mL)*	Clinical Observations**
	Day 0 (10/14/10)	Day 1 (10/15/10)	Weight Change		
DA81/Male	25.6	26.4	0.8	0.51	Normal
DA82/Male	28.2	29.1	0.9	0.56	Normal
DA83/Male	30.1	30.6	0.5	0.60	Normal
DA84/Male	24.1	24.7	0.6	0.48	Normal
DA85/Male	23.2	24.0	0.8	0.46	Normal
Average	**26.2**	**27.0**	**0.7**	**0.52**	
SD	**2.9**	**2.8**	**0.2**	**0.06**	
DA86/Female	26.4	27.1	0.7	0.53	Normal
DA87/Female	28.1	28.9	0.8	0.56	Normal
DA88/Female	33.4	34.3	0.9	0.67	Normal
DA89/Female	27.3	28.2	0.9	0.55	Normal
DA90/Female	27.1	27.6	0.5	0.54	Normal
Average	**28.5**	**29.2**	**0.8**	**0.57**	
SD	**2.8**	**2.9**	**0.2**	**0.06**	

PCE = Polychromatic Erythrocytes
NCE = Normochromatic Erythrocytes
DA = Definitive Assay Animal
MNC = Micronucleated Cells
SD = Standard Deviation
* = Positive Control dosed at 25 µg/g body weight
** = Summary of Clinical Observations, Day 0 through Day 1
@ = Statistically significant increase compared to negative control ($p \leq 0.05$)

TABLE 10
Summary of Micronucleus Assay Results

Test Substance: Solvent Yellow 33 2-(2-Quinolyl)-1,3-indandione,

Lot/Batch #: 0704192/ DOD-D-51485A

Time (Hours)	Dose (mg/kg)	Cell Counts		MNC per 2000 PCE
		PCE	NCE	
24	Vehicle	2000	1966	2.5
24	222	2000	1959	2.8
24	666	2000	2012	2.8
24	2000	2000	1985	3.0
24	CP*	2000	2001	25.6
48	Vehicle	2000	1957	2.8
48	222	2000	1992	2.7
48	666	2000	2004	3.0
48	2000	2000	2019	3.0

10 Animals (5 males and 5 females) were summarized per group.
*CP was used as the positive control and was administered intraperitoneally at 25 µg/g.

APPENDIX I
Software Systems

Software	Use
Adobe Acrobat 8 Professional	Document preparation
DocuKnowledge 3.0	Lotus Domino–based document management system used for SOPs
GraphPad Prism 3	Statistical software for biology studies
Lotus Domino Rel. 5	Client–server application for sponsor, sample, test codes, and quotation management application databases
MS Office 2007 Small Business Suite	Business software (suite includes Word, Excel, PowerPoint, Outlook, Publisher, Office tools)
Rees CentronSQL System 2.0	Environmental monitoring and metrology system
Spiral Biotech QCount 2.2	Plate reader software

ATTACHMENT A
Material Safety Data Sheet

WALRUS ENTERPRISES, LLC
30 Aldrich Street Northampton, MA 01060
Tel: (413) 387-4387 Fax: (413) 586-9359
email: info@coloredsmoke.com
www.coloredsmoke.com

MATERIAL SAFETY DATA SHEET

PRODUCT IDENTIFICATION/DESCRIPTION

Anasol Yellow SG

For emergencies in the US, call CHEMTREC @ 1-800-424-9300

COMPOSITION

A preparation containing solvent dyes

C.I. Name:	**Solvent Yellow 33**
CAS No.:	**8003-22-3**

HAZARDS IDENTIFICATION

As part of good industrial and personal hygiene and of safety practices, avoid all unnecessary, continued exposure to chemical substances, and ensure prompt removal from skin, eyes and clothing.

Eye Contact:	Slightly irritating, if it comes in direct contact.
Skin Contact:	Prolonged, direct contact may cause irritation and sensitization.
Inhalation:	Prolonged exposure may cause upper respiratory irritation.
Ingestion:	Harmful if swallowed.

FIRST-AID MEASURES

Eyes:	Flush eyes with water for at least 15 minutes. Contact a physician.
Skin:	Rinse exposed area with water. Wash thoroughly with soap and water. Contact a physician if irritation develops.
Ingestion:	Contact a physician. Rinse mouth with water. Do not induce vomiting.
Inhalation:	Remove the individual to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Call a physician.

FIRE FIGHTING MEASURES

Flash Point:	NA
Flammable Limits:	ND
Extinguishing Media:	Foam, Dry Chemical, Carbon Dioxide, Water Spray
Special Fire Fighting Procedures:	Protective clothing and self-contained breathing apparatus should be worn when fighting fires involving chemicals
Unusual Fire and Explosion Hazards:	In common with many other organic chemicals, this product may, in certain circumstances, form flammable dust clouds in air.

ACCIDENTAL RELEASE MEASURES

Wear appropriate safety equipment. Stop, contain and cleanup the spill immediately. Contain liquids using absorbents. Sweep up powders carefully, minimizing dusting. Shovel all spill materials into disposal drums and follow all Federal, State and Local disposal regulations.

HANDLING AND STORAGE

Store in a cool, dry, well ventilated area in closed drums. Do not store near heat or ignition sources, or in direct sunlight.

EXPOSURE CONTROLS, PERSONAL PROTECTION

Ventilation:	Use adequate general or local exhaust ventilation
Respiratory Protection:	Dust Mask recommended
Skin Protection:	Wear protective gloves and clothing
Eye Protection:	Wear splash–proof safety glasses or goggles

PHYSICAL AND CHEMICAL PROPERTIES

Boiling Point:	NA	Specific Gravity:	ND
Freezing Point:	NA	Percent Volatile:	ND
Melting Point:	240 ± 5°C	Vapor Density:	ND
Vapor Pressure:	NA	Solubility in Water:	Insoluble

Appearance and Odor: Bright Lemon yellow powder

STABILITY AND REACTIVITY

Stability:	Stable
Conditions to Avoid:	Heat, exposure to open flame.
Incompatibility:	Oxidizing agents
Hazardous decomposition Products:	Oxides of Carbon and Nitrogen
Hazardous Polymerization:	Will not occur

DISPOSAL CONSIDERATIONS

Dispose of in accordance with all Federal, State and Local regulations.

TRANSPORT INFORMATION

DOT Shipping Name:	NA
DOT Hazard Class:	NA
UN/NA Number	NA

REGULATORY INFORMATION

TSCA: All components of this product are registered under TSCA

THE INFORMATION AND RECOMMENDATIONS CONTAINED HEREIN ARE BASED UPON DATA WHICH IS BELIEVED TO BE CORRECT. HOWEVER, NO GUARANTEE OR WARRANTY OF ANY KIND, EXPRESSED OR IMPLIED, IS MADE WITH RESPECT TO THE INFORMATION CONTAINED HEREIN. THIS MATERIAL SAFETY DATA SHEET WAS PREPARED TO COMPLY WITH THE OSHA HAZARD COMMUNICATION STANDARD (29 CFR 1910.1200) THIS SUPERSEDES ANY PREVIOUS INFORMATION.